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Preparation and characterization of monoclonal antibodies against the fifth component of rabbit complement (C5) *

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By immunizing mice genetically deficient in C5 we were able to obtain a group of monoclonal antibodies to rabbit C5 that cross-react with C5 from a wide variety of mammalian sera, including mouse. The specificity of the monoclonal antibodies was against native C5 and C5b but not C5a. The antibodies strongly inhibit the expression of C5 hemolytic activity. We suggest that these monoclonal antibodies will be useful for studying C5 as well as providing a way to selectively deplete C5 from plasma in vitro or in vivo.

Key words: C5; Monoclonal antibody; Complement, rabbit

Introduction

The fifth component of the complement system (C5) is a plasma glycoprotein that is converted to its biologically active forms when cleaved by C5 convertases of the alternative or classical pathway, or by other serine proteases present in injured tissues (Cooper and Müller-Eberhard, 1970; Arroyave and Müller-Eberhard, 1973; Hugli, 1977; Discipio et al., 1981; Wiggins et al., 1981; Sundsmo and Fair, 1985). The fragments, C5a and C5b, are responsible for inducing neutrophil chemotaxis, enhancing the phagocytic, oxygen radical and enzymatic responsiveness of inflammatory cells, and participating in killing through complement membranolytic mechanisms (Cochrane and Müller-Eberhard, 1968; Bhakdi and Tranum-Jensen, 1978; Hugli, 1981; Bult and Herman, 1983). Limited digestion of the molecule with non-complement enzymes can produce two fragments of the α-chain that remain attached to the β-chain by disulfide bonds, giving a molecule with unaltered molecular weight or charge, but with the C5a-like ability to attract neutrophils and C5b-like ability to form a stable complex with C6 (Wetsel and Kolb, 1983).

Individuals who lack C5 are susceptible to recurrent severe bacterial infections (Alper and Rosen, 1984). Larsen and his co-workers showed that C5-deficient mice had lowered survival and recovery when infected with *Pseudomonas aeruginosa*. By contrast, C5-sufficient mice were less hardy than were C5-deficient mice when maintained in hyperoxic conditions, suggesting that C5 contributes to the pulmonary inflammation that occurs during hyperoxia (Larsen et al., 1982; Parrish et al., 1984). Further definition of the role of C5a during inflammation has been hampered by the lack of appropriate reagents for detecting C5 and its activation products in vivo in diverse animal species. In the present paper, we report the development of mouse monoclonal anti-rabbit

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C5 antibodies that cross-react strongly with C5 from a wide variety of mammalian sera. The four antibodies that are described are directed against native C5 and C5b, but not C5a, and have been used to form immunoaffinity beads to study C5 obtained from plasma.

Materials and methods

Immunization of mice, hybridoma production, and ascites collection: four C5-deficient mice (B10.D2/oSn) were immunized because it was hoped that they would respond to rabbit C5 with a broader variety of antibodies than C5-sufficient mice. Each mouse was given 0.1 ml sterile saline containing 52 μg of purified rabbit C5 (Giclas et al., 1981a) intravenously, followed by booster intraperitoneal injections of the same amount of C5 at 1 week, and intravenous injections at 3 and 4 weeks. 4 days after the last injection, the mice were test bled and all four test sera gave single immunoprecipitin lines showing identity with C5 precipitated from rabbit serum by goat anti-rabbit C5. The mice were killed and their spleen cells isolated and fused with P3x63Ag8 mouse myeloma cells to form hybridomas, using the method of Köhler et al. (1975) to isolate cells insensitive to hypoxanthine, aminopterin and thymidine. Successful hybridomas were grown in 96-well tissue culture plates at a starting cell density of 1.5×10^5 cells/well, and cloned by limiting dilution transfer, adding mouse peritoneal cells from a non-immunized animal as feeder cells to promote growth.

An ELISA was used to screen the initial spleen cell colonies and subsequent subclones for antibodies to rabbit C5. The antigen source employed by this assay was either normal rabbit serum (NRS), zymosan-activated rabbit serum (ZARS) containing C5 breakdown products, a 6–11% polyethylene glycol (PEG-4000) cut of NRS that was enriched for C5 concentration, or a mixture of rabbit C3 and C5 obtained as by-products of C5 purification. All the above were diluted in carbonate buffer, pH 9.5, so they contained between 0.8 and 1.0 μg/ml of C5. Rabbit serum albumin (RSA) was used as negative control for the antigens, and an unrelated monoclonal antibody (PLD1), against a rabbit platelet surface antigen, was used as a control antibody. The antigen (100 μl/well) was placed in 96-well microtiter plates (Falcon Microtest III), incubated overnight in a humidified chamber at 4°C, and washed three times with phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20 (PBS-Tween). Buffer containing 2% bovine serum albumin (PBS-BSA) was added to each well and incubated at room temperature for 1 h to block remaining binding sites. The plates were again washed three times with PBS-Tween. Hybridoma supernatants were added to the wells, either undiluted or serially diluted in PBS-Tween, incubated for 1 h at 37°C, and washed three times with PBS-Tween. Alkaline phosphatase-conjugated rabbit anti-mouse IgG (H and L) was added and incubated for 1 h at room temperature, followed by washing three times with PBS-Tween. *p*-Nitrophenyl phosphate, a chromogenic substrate for alkaline phosphatase, was then added, the plate incubated for 1 h at room temperature, and the optical density at 405 nm determined by an ELISA reader (BioTek Instruments, Burlington, VT). Alkaline phosphatase and its substrate were purchased from Bio-Rad (Richmond, CA).

The IgG fraction from the ascites fluid was purified by precipitation with an equal volume of ice-cold saturated ammonium sulfate (SAS). The precipitate was washed in 50% SAS in the cold, and redissolved in a small volume of distilled water. This solution was dialyzed overnight against 20 vols. of 0.01 M phosphate buffer, pH 8.1, and applied to a DEAE-cellulose column (Whatman DE52) previously equilibrated with the same buffer. The column was washed with the starting buffer and the flow-through peak collected. The remaining bound protein was eluted from the column with a linear gradient to 0.5 M NaCl, and fractions were assayed for IgG by immunodiffusion against rabbit anti-mouse IgG (H and L), and for specific antibody by ELISA.

SDS-PAGE analysis employed 7.5% T, 2% C slab gels run under reducing conditions (Laemmli, 1970) and stained with silver stain (Tsang et al., 1984) unless otherwise indicated. Reagents for the polyacrylamide gels were purchased from Bio-Rad (Richmond, CA). Rocket immunoelectrophoresis and immunodiffusion were done by standard methods (Laurell, 1966), using 1% agarose

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(Seakem, ME, FMC Marine Biologicals Division, Rockland, ME). Anti-human C3 and anti-human C5 were obtained from Atlantic Antibodies (Scarbrough, ME) and goat anti-rabbit C3 and anti-rabbit C5 were prepared in our laboratory (Giclas et al., 1981a).

Radioiodination of the monoclonal antibodies with ^{125}I (New England Nuclear, Boston, MA) was catalyzed by Iodogen (Pierce Chemical Co., Rockford, IL), and unbound iodine was removed by a small column of AG-1X8 (Bio-Rad, Richmond, CA) as described elsewhere (Manthei et al., 1984). Typical specific activities achieved by this method were 1.5 μCi/μg protein, with greater than 95% of the radioactivity precipitable by 10% trichloroacetic acid.

Immunoaffinity beads were prepared by coupling the IgG fraction of one of the monoclonal antibodies to cyanogen bromide-activated Sepharose-CL6B (Pharmacia, Upsala, Sweden). Coupling efficiency for the IgG fractions was typically 60–65% of the protein, giving approximately 12–20 mg IgG/ml of beads.

Rabbit, rat, C5-sufficient mouse, C5-deficient mouse, guinea pig, and hamster sera were from animals housed at the investigator's institution. Ferret serum was a generous gift from Dr. Charles Irvin, snake (*Pituophis melanoleucous* and *Thamnophis elegans*) sera were given by Drs. Robert R. Cohen and John Caldwell, and chicken, monkey, and horse sera were from Christina Stevens, all of Denver, CO. Albacore serum was left over from a previous study (Giclas et al., 1981b), and goat and sheep sera were obtained as by-products of antibody production and sheep red blood cell collection.

Rabbit C5 was assayed by rocket immunoelectrophoresis or ELISA to determine its concentration, and hemolytic assays to determine its activity (Giclas et al., 1981a, 1985). A standard pool of NRS was used to calibrate all C5 assays. C5 fragments were generated in vitro by incubation of NRS with zymosan (4 mg/ml), inulin (10 mg/ml) or cobra venom factor (4 U/ml).

Results

Four clones were chosen for production of ascites fluid in BALB/C × B10.D2/oSn F_1 mice. Hybrid mice were used because the hybridomas were derived by fusion of cells from both strains. Ascites fluids were collected and pooled, and the IgG fractions isolated.

An ELISA titration was done with the first monoclonal antibody (CWD1) against NRS, PEG-RS, C3/C5 and RSA, diluted so that the C5 concentration in each of the first three was 0.8–1.0 μg/ml, and the RSA concentration was the same as that in diluted NRS. Equivalent binding was obtained to all of the C5-containing antigen sources (Fig. 1) and no binding to RSA occurred. Similar results were obtained with the other three monoclonal antibodies. Table I lists results from two sets of experiments that were done as follows: (1) ELISA titration was performed, using C3/C5 as the antigen source, and the monoclonal anti-C5 antibodies and the unrelated monoclonal antibody (PLD1) were compared for their binding capacities. The results were calculated as the log of the antibody concentration (ng/ml) versus the percent of maximum color development in the ELISA, and the curves analyzed by linear regression to obtain the concentration at which 50% of the

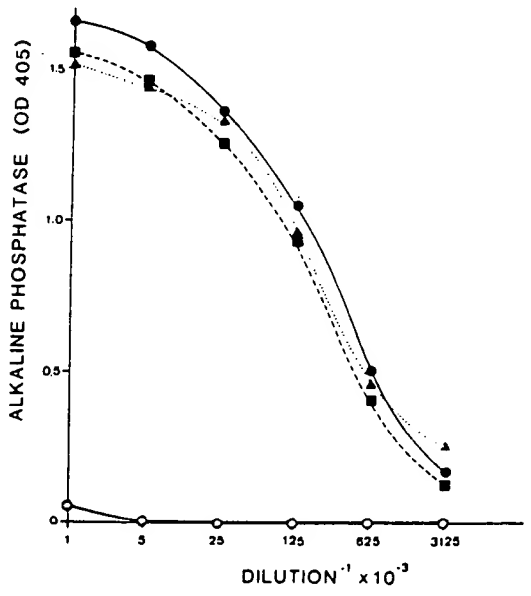


Fig. 1. ELISA titration of CWD1 monoclonal anti-rabbit C5 ascites fluid against NRS (■), PEG-RS (●), C3/C5^{rab} (▲) and RSA (○), as described in the text. C5 concentrations of the first three antigens were 0.8–1.0 μg/ml, and CWD1 ascites protein concentration was 70 mg/ml.

TABLE I

AMOUNT OF MONOCLONAL ANTIBODY (PURIFIED IgG) NEEDED TO ACHIEVE 50% OF MAXIMUM RESPONSE IN ELISA, OR 50% INHIBITION OF HEMOLYTIC C5 ACTIVITY

Antibody used	IgG concentration (ng/ml)	
	ELISA	C5H50
CWD1	110.3 ± 14.5 (7:1) [a]	560 ± 75 (1:6.2)
CWD2	20.9 ± 0.8 (38:1)	333 ± 76 (1:3.7)
CWD3	13.8 ± 0.6 (58:1)	50 ± 13 (1:0.6)
CWD4	20.3 ± 1.2 (39:1)	907 ± 154 (1:10.1)
PLDI	[b]	1.08×10^8 ($1:1.2 \times 10^6$)

[a] Mean ± SD (antigen : antibody ratio, w/w)
[b] PLDI was less than or equal to background with no antibody.

maximum response occurred. (2) Inhibition of C5 hemolytic activity was measured in samples of the NRS pool diluted 1:1000 in buffer containing 0.01 M EDTA, giving approximately 80 ng/ml C5. EDTA does not inhibit the assay for C5, but was used to block possible activation of complement by C5-anti-C5 immune complexes. The NRS-EDTA dilutions were preincubated for 30 min at 37°C with serial dilutions of each of the anti-C5 monoclonals and PLD1, and C5 titers were determined by hemolytic assays. CWD3 showed the greatest inhibition of C5 hemolytic activity per ng of IgG, and also had the highest titer in the ELISA.

In order to determine whether any of the four antibodies reacted with the C5a fragment of rabbit C5, a preparation of rabbit C5 fragments (C5f) was prepared from zymosan-activated rabbit plasma by the method of Fernandez and Hugli (1976), with substitution of molecular sieving through a membrane with a nominal M_r 30000 cut-off (Centricon-30, Amicon, Lexington, MA) for the gel filtration column. The resulting rabbit C5f preparations had specific activities equivalent to highly purified human C5a, when tested for their ability to induce enzyme release from cytochalasin B-treated human neutrophils (Webster et al., 1980), and to induce vascular hyperpermeability in rabbit skin (Cochrane and Müller-Eberhard, 1968). Because it was likely that the ascites fluid contained carboxypeptidase N activity that might inactivate the C5f (Vallota and Müller-Eberhard, 1973), the antibodies to be used were pretreated with the arginine analogue, MGETPA, (DL-2 mercaptomethyl-3-guanidinoethyl-thiopropionic acid, Calbiochem, La Jolla, CA), a competitive carboxypeptidase inhibitor (Plummer and Ryan, 1981). Aliquots of the C5f preparation were incubated for 30 min at room temperature with buffer or with dilutions of the MGETPA-treated CWD antibodies, and then assayed for their ability to induce myeloperoxidase release from human neutrophils (Webster et al., 1980). Table II gives data from an experiment in which there was no significant difference from the activity of C5f when incubated with CWD1, PLD1, or no antibody. None of the monoclonal antibodies had anti-C5a activity when tested by this method. In another set of experiments, the rabbit C5f preparation was incubated with immunoaffinity beads containing either the monoclonals, anti-human C3 or anti-human C5 for 30 min at room temperature in buffer containing 1 M MGETPA. The immunoaffinity beads were then removed from the C5f by centrifugation, and remaining activity assayed as above. There was no decrease in the MPO titer of the C5f after treatment with any of the immunoaffinity beads.

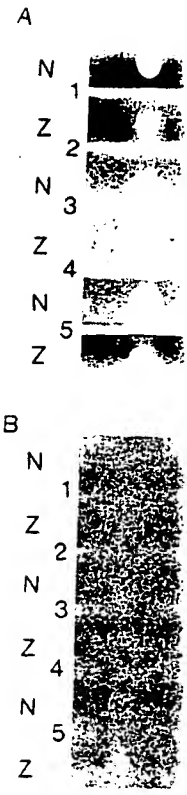
Since none of the monoclonal antibodies formed precipitin lines on immunodiffusion against rabbit serum, each was radiolabelled with ^{125}I and mixed with a 'carrier' antiserum (goat anti-human C5, or anti-whole rabbit serum) so that each mixture contained approximately 1×10^5 cmp/μl. Results of immunoelectrophoresis of NRS and ZARS are shown in Fig. 2. As can be seen from the auto-

TABLE II

SECRETOGOGUE ACTIVITY OF RABBIT C5f AFTER INCUBATION WITH MONOCLONAL ANTI-C5, OR PLDI, AN UNRELATED MONOCLONAL

Antibody dilution	Enzyme release [a]	
	CWD1	PLDI
1/10	16.5 ± 9.5	51.7 ± 18.9
1/100	87.8 ± 14.3	114.3 ± 13.6
1/1000	101.2 ± 26.8	109.0 ± 1.6
1/10000	93.3 ± 7.2	128.7 ± 27.2

[a] Percent of maximum release induced by C5f (35% of total MPO in neutrophils) (mean ± SD, $n = 3$).



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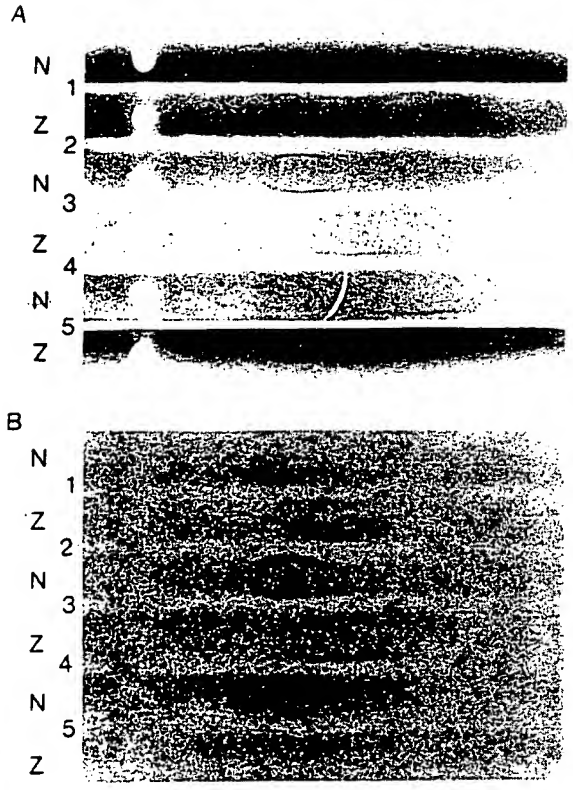

Fig. 2. *A*: Immunoelectrophoresis gel stained with 1% amido black. Wells marked N contained normal rabbit serum, those marked Z contained zymosan-activated rabbit serum. Troughs 1–4 contained goat anti-rabbit C5 mixed with ^{125}I-CWD1, -2, -3, or -4, respectively, and trough 5 contained an equal amount of ^{125}I-CWD4 mixed with anti-rabbit serum albumin. *B*: Autoradiogram of the same gel.

radiograms, all four CWD antibodies coprecipitated with the anti-C5 against both C5 and C5b. Only one arc, corresponding to the position of C5, was radiolabelled when anti-whole rabbit serum was used. CWD4 was mixed with anti-RSA in the last trough, and no radiolabel was coprecipitated. In parallel experiments, each of the monoclonals was mixed with a variety of antisera, including anti-C3, anti-C4, and anti-IgG, but none of the CWD antibodies coprecipitated with any antibody except anti-C5.

A further experiment was done to verify that the CWD antibodies were specific for C5. Immunoaffinity beads were prepared with each monoclonal, and with polyclonal anti-human C3 and anti-human C5. A small aliquot of each of these affinity beads was incubated with NRS in the presence of 0.01 M EDTA, and washed exhaustively with 0.02 M Tris pH 7.4, containing 0.15 M NaCl and 0.1% Tween 20 to remove non-specifically bound protein. The beads were then washed with 3.8% sodium thiocyanate to elute the bound antigen, and this was reduced and analyzed by SDS-PAGE. The gels were silver stained and scanned by a soft laser 2D scanning densitometer (Biomed Instruments, Livermore, CA). The first gel was run with samples precipitated by the four monoclonals along with anti-human C3 immunoaffinity beads. The superimposed tracings shown in Fig. 3*A* were from protein eluted from CWD3 and anti-C3, and it can be seen that the α chains were different. The superimposed tracings shown in Fig. 3*B* were from protein eluted from CWD3 and anti-C5. The molecular weights of the proteins eluted from all four CWD antibodies were the same as C5 α and β chains, and were different from C3. The lower bands on the gel are albumin (67 kDa) and the heavy chain of mouse IgG (50 kDa), both of which were contaminants on all of the gels. Preadsorption of the NRS sample by beads containing goat anti-C5 depleted the protein bound by the CWD beads but preadsorption with anti-C3 beads did not. Further proof that the antigen was C5 was obtained from the gel shown in Fig. 4, in which normal and C5-deficient human sera and mouse sera were precipitated with the immunoaffinity beads. The anti-C3 immunoaffinity beads brought down two-chain C3 protein from all four sera, whereas the CWD4 immunoaffinity beads brought down two-chain C5 protein only from the two normal sera and not from the C5-deficient sera.

To determine the cross-reactivity of the monoclonals with C5 of different species, sera were obtained from the following animals: rabbit, rat, guinea pig, hamster, mouse, dog, cat, ferret, monkey, human, goat, horse, cow, sheep, tuna, bull snake, garter snake, and chicken. Immunoprecipitates were made from each of these with the immunoaffinity beads, and analyzed on silver-stained polyacrylamide gels. The proteins precipitated from all of the mammalian sera had two chains with molecular weights similar to those of rabbit C5. Fig. 5 shows a gel that was scanned and the molecular weights of the α and β chains

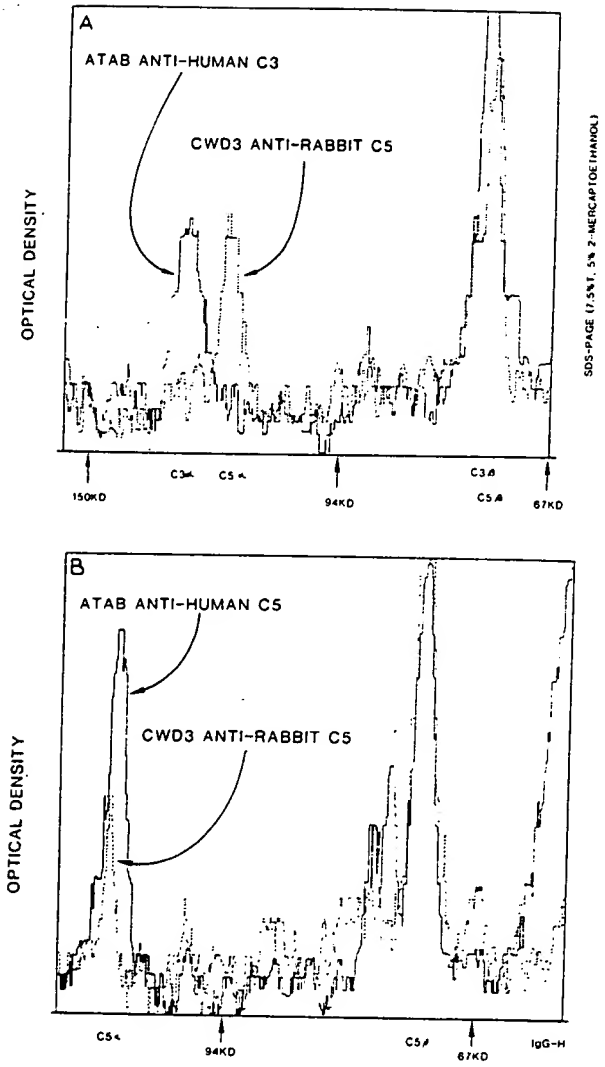

Fig. 3. *A*: Scanning densitometer tracing of a polyacrylamide gel lane containing protein immunoprecipitated from normal rabbit serum using immunoaffinity beads with goat anti-human C3 (solid line) superimposed on tracing from the adjacent lane containing protein immunoprecipitated with CWD3, as described in the text. Positions of the C3 and C5 α and β chains are shown on the bottom, along with molecular weight marker positions. *B*: Densitometric tracing of lanes containing proteins precipitated from rabbit serum by anti-human C5 (solid line) or CWD3.

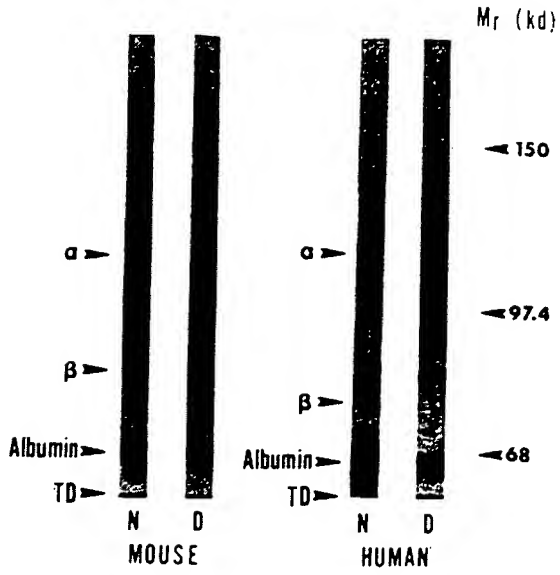

Fig. 4. Polyacrylamide gel analysis of normal and C5-deficient mouse serum (N and D) and normal and C5-deficient human serum (N and D), immunoprecipitated with immunoaffinity beads containing CWD4 monoclonal anti-rabbit C5. Positions of molecular weight standards are shown at right. The protein at M_r 67000 in all four lanes is albumin, a frequent contaminant of the immunoprecipitates. The protein at M_r 80000 in the lanes from the two human sera has not been identified, but was also present in lanes from control immunoaffinity beads with anti-C3.

were as follows: human = 113720 and 76049, ferret = 113111 and 75641, dog = 119631 and 82427, rabbit = 117089 and 77284, mouse = 113415 and 83317, hamster = 113835 and 86074, rat = 118353 and 81986, guinea pig = 115102 and 83261. The albacore, snake, and chicken sera did not produce two distinct chains, but had broad bands at approximately 70–80000. Whether these are related to C5 has not been ascertained.

Experiments to define the binding site of the monoclonals indicate that it is present only on undenatured, non-reduced C5 or C5b. This is demonstrated in Fig. 6. Aliquots of NRS were incubated with the complement activators zymosan, inulin, and cobra venom factor, then immunoprecipitated and examined by SDS-PAGE. However, if the antigen was denatured or reduced on SDS-polyacrylamide gels and Western blot analysis done with the monoclonal antibodies, no specific binding could be detected. Purified rabbit C5 was treated with 0.2 M mercaptoethanol (1 h, room temperature) followed by alkylation with 0.3 M Iodoacetic (pH 7.5, 1 h on ice). The reduced and alkylated protein was incubated with immunoaffinity beads as above, and the bound pro-

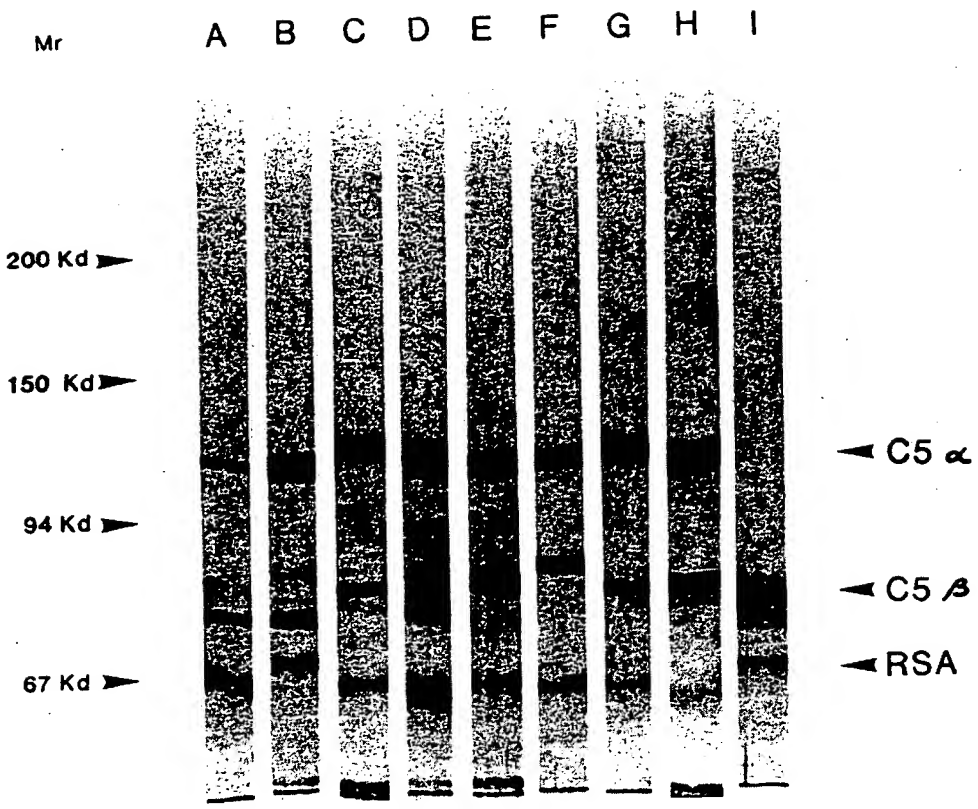

Fig. 5. Polyacrylamide gel showing proteins immunoprecipitated from sera of nine animals species: (A) human, (B) ferret, (C) dog, (D) rabbit, (E) mouse, (F) hamster, (G) rat, (H) guinea pig, and (I) albacore (left to right). Albumin can be seen below the β chains in each lane, as well. The approximate positions of C5 α and C5 β are marked on the right side, and the molecular weight standards are shown at left.

tein analyzed on polyacrylamide gels. The gel lane containing reduced and alkylated C5 control had two bands at the C5 α and C5 β positions, but the lanes containing protein eluted from the immunoaffinity beads were blank, with the exception of those from CWD4 that had faint protein bands in the C5 β position.

Discussion

Four monoclonal antibodies that were prepared against rabbit C5 have been described. The identity of the antigen recognized by these antibodies was confirmed by several means. First, radiolabelled monoclonal antibodies coprecipitated only with antisera against rabbit or human C5, and not with antisera against C3, C4, albumin, or any other plasma proteins. Second, the protein immunoprecipitated from rabbit serum with immobilized monoclonal antibodies consisted of two polypeptide chains with the same molecular weights as those of C5, and was clearly different from C3. Third, no protein could be precipitated from C5-deficient human or mouse sera, whereas C3 was precipitable from both normal and C5-deficient sera with anti-human C3 immunoaffinity beads. Fourth, the monoclonals all recognized both native C5 and C5b, but not C5a.

Our original idea that the C5-deficient mouse was immunologically naive to C5 and therefore more likely to make antibodies with fewer species-restricted specificities was borne out. Broad cross-reactivity was shown by the ability of the

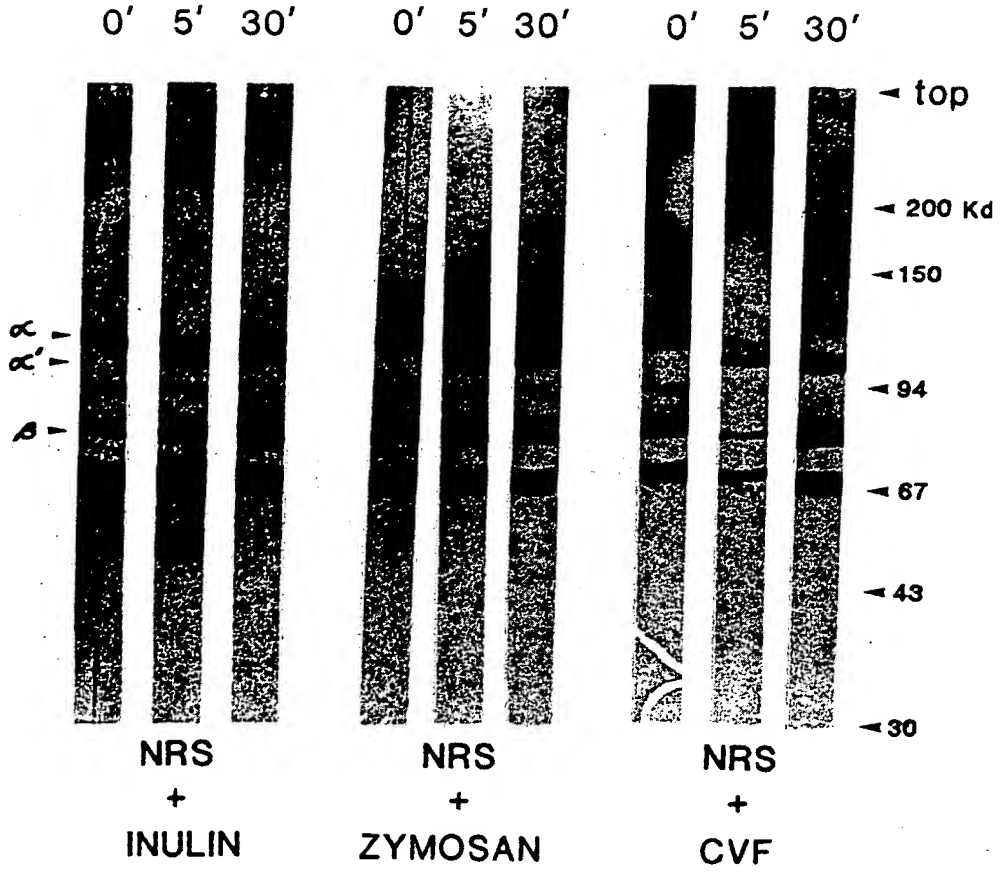

Fig. 6. Polyacrylamide gel analysis of C5 protein precipitated from rabbit serum at time 0, 5, and 30 min after initiation of complement activation with inulin (10 mg/ml), zymosan (4 mg/ml) and cobra venom factor (4 U/ml). The appearance of the C5 α' chain (104 136 ± 436 Da) can be seen at 5 and 30 min.

monoclonals to precipitate C5 from the sera of a wide variety of mammalian species, but not from sera of the non-mammalian vertebrates tested to date, suggesting that they recognize relatively well conserved configurations of C5. The exact binding sites of the monoclonals have not yet been determined. They all bound C5 from normal serum and C5b from activated serum. Attempts to probe Western blots of reduced C5 with ^{125}I-CWD antibodies were negative, and when purified C5 was reduced and alkylated in solution, the immunoaffinity beads were no longer able to precipitate protein. Thus, the conformational changes that accompany denaturation appear to make the binding site unavailable.

Because these monoclonal antibodies react with C5 from many different species, they should provide useful tools for studying C5 activation in vivo and in vitro, as well as providing a way to selectively deplete animal sera of C5 and thus create homologous assay systems for measuring C5 hemolytic activity in these species, or, using F(ab′) fragments, to temporarily deplete C5 in vivo.

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